1973

Mitochondrial behavior during the respiratory climacteric and ripening of detached apples.

Wing-Yee Chan
University of Massachusetts Amherst

MITOCHONDRIAL BEHAVIOR DURING THE RESPIRATORY CLIMACTERIC AND RIPENING OF DETACHED APPLES

A Thesis Presented

By

WING-YEE CHAN

B.Sc., Chung Chi College, the
Chinese University of Hong Kong

Submitted to the Graduate School of the
University of Massachusetts in partial
fulfillment of the requirement for the degree of

MASTER OF SCIENCE

Department of Plant and Soil Sciences
University of Massachusetts

July 1973

C O N T E N T S

LIST OF TABLES

A P P E N D I X

L I S T O F F I G U R E S

I N T R O D U C T I O N

The respiratory climacteric of fruit is a characteristic rise in the rate of respiration during fruit ripening. It was observed by Kidd and West (34) in the early 1920's while studying the changes in respiratory activity of 'Bramley's Seedling' apples picked at various stages of growth. They found that the CO_2 production of fruit fell to a minimum value and then rose rapidly and subsequently respiration declined, and that fruit quality (color, taste, and texture) reached its peak at or near the respiratory peak. Kidd and West (34) recognized that the increase in respiration was a critical phase in the life of the fruit and hence termed it "the climacteric". Biale (5) concluded that this relatively sudden alternation in the level of respiration marked the transition from growth to the senescence phase in the life of fruit.

Not all types of fruit exhibit a climacteric pattern during ripening. Those that do possess a climacteric pattern of development are called climacteric fruits. Among these are apples, apricots, avocados, bananas, cantaloupes, melons, mangoes, papayas, passion fruit, peaches, pears, plums and tomatoes (4,51). Those that do not exhibit the climacteric phenomenon are called non-climacteric fruits. Under this category are cherries, cucumbers, figs, grapes, grapefruit, lemons, oranges, pineapples and strawberries

(4, 51). For these fruits, the respiratory pattern shows a slow drift downward after detachment from the parent plant.

Considerable research has been conducted on the nature and control of the respiratory climacteric, yet the phenomenon is still not clearly understood. One area of uncertainty is the role played by mitochondrial changes during the climacteric. In view of their importance as the site of cellular respiration, the activities and physical properties of mitochondria could be expected to be closely related to the respiratory climacteric. It was the intent of this investigation to study changes in activities and physical properties of isolated mitochondria during the respiratory climacteric rise and fall of 'Delicious' apples.

Specific aims were:

1. To compare the CO_2 production of whole fruit with the activity of mitochondria on the substrates succinate, malate, pyruvate and NADH, at different stages of the climacteric.
2. To examine the effect of thiamine pyrophosphate (TPP), arsenite and glutamate on mitochondrial oxidation of various substrates during fruit ripening.
3. To observe the changes in swelling and contraction capacity of the mitochondria during the climacteric.

L I T E R A T U R E R E V I E W

1. The Climacteric.

The climacteric pattern of respiratory changes was demonstrated in very early experiments of Gerber (16) in 1897. However, it was not until the studies of Kidd and West (34) that the significance of the respiratory rise in relation to the process of fruit ripening was recognized. Subsequently, a respiratory peak was shown for tomato by Gustafson (17), for avocado by Wardlaw and Leonard (59), and for banana by Leonard (40). In 1960 Biale (4) classified fruits into climacteric and non-climacteric categories. There is no logical distinction between the kinds of fruits in the two groups, except that citrus all seem to be non-climacteric. Absolute classification of fruits in the non-climacteric category is difficult, because it is possible that at a more appropriate stage of development a respiratory climacteric might be displayed. In fact, melon was originally classified as non-climacteric (4), but it has since been shown to exhibit the typical climacteric pattern by Lyons and McGlasson (45). Spencer (57) believes that the same mechanism of ripening occurs in both groups of fruit except that events occur slowly over a long period in the non-climacteric type, and are telescoped in the climacteric fruit into a short dramatic period.

There are two general interpretations of the respirat-

ory climacteric in fruits. The first one is put forth on the basis of physical changes, involving an increase in permeability allowing substrates access to existing enzymes and thereby leading to enhanced metabolism. Sacher (55) in 1966, working on banana pulp, tried to determine whether there was an increase in permeability of cells preceding the onset of the respiratory climacteric. He found an increase during the climacteric rise, to almost 100% free space to chloride, mannitol and sucrose. However, his findings were challenged by Burg (9) who showed that banana pulp cells at the respiratory peak were capable of plasmolysis and the maximum estimate of free space was below 60%. Various other workers (8, 28) also challenged this theory that the climacteric is caused by changes in membrane permeability. However, Hulme (28) did suggest that small selective changes in permeability may be involved in the early process of ripening.

The second theory is based on changes in the pattern of protein synthesis in which new enzymes are synthesized to catalyse the ripening process. Hulme (24) in 1954 found that there was an increase in protein nitrogen content of apples as they entered the climacteric phase. An increase in the activity of NADP-dependent malic enzyme and pyruvate decarboxylase were found in apples during the climacteric rise (25), thus accounting for the increase in Respiratory

Quotient (i.e. the output of CO_2 divided by the uptake of O_2). Decarboxylation of malate to give acetaldehyde and ethanol through pyruvate metabolism presumably contribute to the increase in CO_2 production. Since the increase of malate decarboxylating system is greater than that of the pyruvate decarboxylating system, this increase in CO_2 is called the "Malate Effect"(25).

Chloroplast lamellae have been found to disintegrate during the climacteric and the constituents of the membranes were believed to be used as building materials in the new enzyme synthesis (2). Hulme et al. in 1968 (28) showed that enhanced RNA and protein synthesis is associated with the climacteric in apple. Similar results were also found for avocado (54), pear (15) and banana (8). By using a polyacrylamide gel electrophoresis technique Frenkel et al. (15) also found that only a relatively specific group of proteins was synthesized at the early stage of climacteric, and in particular, malic enzyme. These studies strongly suggest that the synthesis of new enzyme protein causes a rise in metabolic activity during the climacteric.

2. Mitochondrial Activities During The Climacteric.

As mitochondria are directly involved in respiratory activity, their activity has been widely studied in relation to the respiration climacteric. Pearson and Robertson (50) in 1954 were the first to obtain a crude mitochondrial

fraction from fruit using 'Granny Smith' apples. They found evidence that an increase in mitochondrial activity occurred as the fruit passed through the climacteric. Their work, together with later work by Lieberman (42) and Hatch et al. (20), showed that a Tricarboxylic Acid (TCA) Cycle and a cytochrome oxidase system operated in apple. Haard and Hultin (18) also showed that banana mitochondria were capable of oxidizing various TCA cycle intermediates.

Whether or not mitochondrial activity increases during the climacteric is a subject of debate. Hulme et al. (26) in 1964 obtained mitochondrial fractions from the peel of apples having 30 times the activity of the original preparations of Pearson and Robertson (50), and they reported an increase in activity of mitochondria isolated from fruits at succeeding stages of the respiratory rise. Similar results were found by Jones et al. (32) for apples both on and off the tree, although the climacteric on the tree proceeded more slowly and rose to a higher peak. However, Lance et al. (36) detected no increase in activity of mitochondria during the climacteric of avocados. For tomatoes, also, Hobson (22) found that the mitochondria appeared to be at their most active state from fruit shortly before the climacteric (more or less equivalent to the mature green stage). Particles separated from tomato fruit at stages up to full ripeness showed a declining ability

to oxidize succinate, malate and α-ketoglutarate. The results of Lance et al. (36) and Hobson (22) suggested that co-factor availability might account, at least in part, for the climacteric rise in respiration.

A. Factors That Might Account for An Increase of Mitochondrial Activity during The Respiratory Climacteric.

An early hypothesis to explain the respiratory climacteric was that mitochondrial oxidation becomes progressively uncoupled from phosphorylation during fruit ripening. Millerd et al. (49) using the uncoupling agent dinitrophenol (DNP) on avocado tissue slices found that the respiration of preclimacteric fruit slices could be stimulated by application of DNP, while slices from the climacteric peak fruit were not stimulated by such treatment, so they suggested that the ripening fruits contained a natural uncoupling substance like DNP which increased respiration and accounted for the climacteric rise. Later workers, however, have shown that both intact fruit and extracted mitochondria at and after the climacteric still possess an ability for phosphate esterification (48), a high P/O ratio (33) and a high respiratory control ratio (60). The Uncoupling Hypothesis has been largely ruled out by Young and Biale's work (61) in 1967 using P^{32} incorporation into avocado slices. They found that though the rate of uptake of P^{32} was greater in the climacteric tissue,

94% of P^{32} was lost from the climacteric tissue while only 31% was lost from the preclimacteric tissue during washing; it was the technique in washing that caused the "uncoupling effect" observed by Millerd *et al*. (49).

A more recent consideration is the possibility that an increase in mitochondrial activity might result from an increased synthesis or availability of essential co-factors involved in the respiratory pathways, e.g. the pyridine nucleotides NAD and NADP. It was shown by Rhodes and Wooltorton (53) in 1968 that the concentrations of NAD and NADP (both oxidized and reduced forms) are relatively high throughout the development of the apple with no major or significant change occurring during the period of climacteric. They concluded that it is unlikely that the onset of the climacteric is limited by the availability of pyridine nucleotides.

Another co-factor recently studied as a factor controlling the respiratory rise is thiamine pyrophosphate (TPP). It had been shown that the replacement of the yeast extract used in mitochondrial assay medium by a mixture of ATP, NAD, NADP, TPP and CoA had little or no effect on the activity of the mitochondria from mature apples before or after the climacteric peak (29). However, Lance *et al*. (37) found that with avocados the oxidation of malate by mitochondria in the preclimacteric phase was markedly

stimulated by TPP. Once the climacteric phase was entered, the response of mitochondria to TPP declined. Hobson (22) also found mitochondrial stimulation by TPP during the climacteric using tomato fruit except that the response to additional TPP during malate oxidation persisted with tomato mitochondria toward senescence.

The pronounced stimulatory effect of TPP on mitochondria from preclimacteric fruit was thought to relate to the accumulation of the inhibitor oxaloacetic acid (OAA) in the mitochondria (29). Addition of TPP could stimulate pyruvate oxidation and dissipate the excess OAA. TPP stimulation of malate and pyruvate oxidation was prevented by arsenite, an inhibitor of keto-acid oxidation (36, 37). The addition of glutamate, on the other hand, increased the rate of malate oxidation, possibly through transamination of OAA (29). Lance et al. (37) suggested that an increased availability of TPP could play an important role in initiating the climacteric, by removing OAA inhibition of respiration. However, Hulme et al. (29) working with apples, found an increase in transaminase activity of mitochondria during the development of the climacteric. Therefore, they thought that removal of OAA in mitochondria involved mainly transamination rather than TPP stimulation of pyruvate oxidation.

Mitochondrial activity might increase during the

climacteric due to an increase in mitochondrial number as well as to an increase in activity of a preexisting mitochondrial system. Hulme (24) in 1954 found that there was a net increase in protein nitrogen (i.e., nitrogenous material insoluble in 80% ethanol) during the climacteric. This might be due at least in part to an increase in mitochondrial number. Lance and Bonner (35) showed for a number of plant tissues (but unfortunately, not including fruits) that the number of mitochondria per cell is the controlling factor in the intensity of tissue respiration. Hulme *et al*. (28) in their preliminary electron microscope studies observed a relative abundance of dumbell-shaped profiles early in the climacteric phase, which might indicate mitochondrial multiplication. But other workers (24) showed that the rate of respiration per unit protein nitrogen, i.e. R/P ratio, was constant from the end of cell division to the onset of the respiratory climacteric. During the climacteric of apples the R/P ratio rose considerably, suggesting that there is no simple relationship between respiratory oxidation and protein synthesis (24). Moreover, recent work of Richmond and Biale (54) using puromycin on avocado, Frenkel *et al*. (15) using cycloheximide on pears and Brady *et al*. (8) using cycloheximide and fluorophenylalanine on banana, showed that inhibitors of protein synthesis had no effect on the respiratory

climacteric. This would suggest that the increase in respiration was independent of protein synthesis and hence independent of an increase in mitochondrial number. The question of increase in mitochondrial number as related to an increase in mitochondrial activity remains unresolved and requires further study.

B. Swelling and Contraction Properties of Mitochondria.

Swelling and contraction of animal mitochondria have been extensively investigated, and it is established that within the limits imposed by their extensibility and compressibility mitochondria behave as osmometers, and that they are relatively permeable to salts such as KCl, but much less so to sucrose and other polyhydroxy compounds. Osmotic adjustment is obtained within a few seconds on changing osmolarity, but there is a slow swelling (spontaneous swelling) which can be accelerated by a wide variety of swelling agents, and which is largely reversible by ATP or phosphorylating respiration. The swelling is somehow linked to electron transfer, since inhibitors of respiration prevent swelling (38).

Relatively little work has been done on swelling and contraction of plant mitochondria, but it appears that they behave differently from animal mitochondria. Investigations have been carried out on the relationship between swelling and succinate oxidation of lupine mitochondria

(23), on the effect of ethylene on volume changes of cauliflower mitochondria (46), and on the relationship between mitochondrial swelling and the chilling resistance of plant tissue (47). However, the majority of work has been done by Hanson and his associates using corn mitochondria (19, 58).

Stoner and Hanson (58) observed a rapid spontaneous swelling in buffered KCl, and found that contraction could be initiated or maintained by substrate oxidation or ATP hydrolysis in the presence of Mg^{++}. Substrate-induced contraction was inhibited by inhibitors of electron transfer, and ATP-induced contraction by oligomycin. They suggested that the degree of contraction is associated with the level of a non-phosphorylated high energy intermediate of oxidative phosphorylation, X~I, and swelling is the result of spontaneous hydrolysis of X~I. Small concentrations of inorganic phosphate inhibit contraction, especially when substrate-induced. This is thought to be due to the production of phosphorylated intermediate X~P, thus lowering the level of X~I. Similar results were obtained by Earnshaw and Truelove (14) with Phaseolus hypocotyl mitochondria. They found that the conditions under which the mitochondria are swollen affect subsequent contraction and substrate oxidation, but not their oxidative phosphorylation ability. Bovine serum albumin (BSA) was found to

reduce the rate of swelling and to promote substrate oxidation, contraction and ion accumulation. They suggested that swelling of mitochondria is associated with the release of malic dehydrogenase and a loss of membrane integrity, which may reflect a change in its permeability.

No previous determination of mitochondrial swelling or contraction during the respiratory climacteric has been made. Indeed, the whole question of the significance of these phenomena to plant development is unknown.

3. Criteria for Intact Mitochondria.

In order to determine whether isolated mitochondria are intact or not, there are several criteria or properties which isolated mitochondria must exhibit to assume that they are reasonably intact and have maintained their morphological integrity. Lehninger (38) has shown that high respiratory control and phosphorylation are good measures of mitochondrial intactness. Also, highly active tightly-coupled mitochondria are associated with low ATPase activity (6, 21) which can be shown with the addition of ADP as follows. The rate of oxidation is initially stimulated by the addition of ADP; if there is low ATPase activity the rate of oxidation will return to its non-stimulated state at the exhaustion of the ADP. In the terms of Chance and Williams (10, 11), the stimulated condition is referred to as State III, and the rate after exhaustion of ADP is

referred to as State IV.

Bonner (7), working with plant mitochondria, and Hedman (21), working with animal mitochondria, reported that damaged mitochondria lose cytochrome c and that this is manifested in cytochrome c stimulation of oxidation of various substrates. Although most studies indicate that the fundamental structure and function of plant and animal mitochondria are alike, some apparent differences have been found. It had been found that the inability to respire added NADH in animal mitochondria indicated a degree of membrane integrity and purity of mitochondria. However, Ikuma and Bonner (31) working with higher plant mitochondria, found that they readily oxidized added NADH and their ability to respire added NADH is one of the major differences between plant and animal preparations.

M A T E R I A L S A N D M E T H O D S

1. Source of Fruit.

'Delicious' apples were harvested on October 4, October 17, and October 30, 1972 from trees at the Horticultural Research Center, Belchertown. On each harvest date, fruits were carefully selected by color so that they would be of uniform maturity as possible. These harvest dates appeared to provide fruit of 3 distinctly different stages of maturity. After harvest the apples were stored at $0^{o}C$, 95% relative humidity, until use. At the time they were used the lots were found to be at the following stages of development: October 4 harvest, climacteric rise; October 17 harvest, climacteric peak; October 30 harvest, post-climacteric. To provide a fourth developmental stage, apples from the October 30 harvest were placed at room temperature for 2 weeks to give a stage of advanced senescence. These 4 lots of fruit were used to determine mitochondrial differences at the 4 specified developmental stages. During the experiment the fruit were maintained at $0^{o}C$, 95% relative humidity.

A second experiment was conducted to follow changes during sequential ripening of a single lot of fruit. For this time course experiment, 'Richared Delicious' apples were obtained 3 months after harvest directly from Controlled Atmosphere (CA) storage (3% O_2, 3% CO_2, $0^{o}C$, 95%

relative humidity) at the Horticultural Research Center, Belchertown.

2. Isolation of Mitochondria.

Apple mitochondria were isolated by Shipway's method (56) which involved gentle grating in an extraction medium followed by straining and differential centrifugation. The extraction medium was composed of : sucrose, 0.4M; citrate (Na), 0.02M; KH_2PO_4, 0.01M; polyvinyl-pyrrolidone (PVP)-pharmaceutical grade, 0.75% (w/v); ethylenediamine tetra-acetic acid (EDTA), 0.01M; and cysteine hydrochloride, 0.01M, added immediately prior to extraction. The medium was maintained at pH 7.8 throughout the extraction procedure by dropwise addition of 20% KOH, monitored by a Beckman Model G pH meter. The medium was continuously stirred by a magnetic stirrer. All operations were carried out at 4°C to 6°C and all apparatus was pre-chilled. Care was taken to insure that the grating surface was maintained below the surface of the extraction medium so that once the cells were disrupted the contents were immediately bathed in buffered extraction medium. About 400-500 g of tissue were grated into 750 ml of medium. The resulting extract was strained through 4 layers of cheese cloth and then through a 50 u mesh nylon fabric (Size D, General Biological Co., 8200 S. Hayne Ave., Chicago, Ill.) to remove the major portion of starch present within the extract. The filtrate

was then centrifuged for 10 min at 37,000g (SS-34 rotor) in a refrigerated Sorvall RC2B centrifuge. The green mitochondrial pellet obtained, together with some adhering starch, was washed using a Thomas homogenizer (glass-teflon, size C) in 30 ml of wash medium containing sucrose, 0.4M; EDTA, 0.01M; and trishydroxymethylaminomethane (Tris), 0.1M, with pH adjusted to 7.5. The homogenate was centrifuged for 10 min at 1,000g to remove any adhering starch from the mitochondrial fraction. The pellet which resulted from the centrifugation at 37,000g for 10 min was suspended in 1 ml of sucrose, 0.2M (pH 7.2) using a Thomas homogenizer (glass-teflon, 5 ml size). The mitochondrial preparation was brought up to a total volume of 2 ml. The total extraction time varied between 1½ and 2 hr. The extraction procedure is schematically illustrated in Figure 1.

An attempt was made to purify the isolated mitochondria using the method of Douce et al. (13) with some modifications. The washed mitochondria were layered on top of discontinuous sucrose gradients and centrifuged in a 30 rotor (Beckman Model L preparative centrifuge) for 1½ hr at 29,000 rpm (≈80,000g). The gradients were prepared by layering sucrose solutions containing 0.1% BSA and 10mM phosphate buffer into centrifuge tubes in the following sequence of concentrations: 1.8M (6 ml), 1.45M (6 ml), 1.2M (6 ml), 0.9M (3 ml), 0.6M (3 ml) and 0.4M (3 ml).

TISSUE GRATED INTO ISOLATION MEDIUM,
STRAINED THROUGH 4 LAYERS OF CHEESECLOTH

DISCARD PULP

FILTRATE
Strained through nylon fabric
(50u mesh)

DISCARD PRECIPITATE

FILTRATE
Centrifuge: 37,000g for 10 min

DISCARD SUPERNATANT

Suspend pellet in wash medium
Centrifuge: 1,000g for 10 min

DISCARD PELLET

SUPERNATANT
Centrifuge: 37,000g for 10 min

DISCARD SUPERNATANT

MITOCHONDRIAL PELLET
Suspend in 0.2M sucrose

Figure 1. Flow-chart for the isolation of mitochondria from apple tissue.

Following centrifugation the mitochondria were found at the boundary between 1.2 and 1.45M sucrose. The mitochondria were removed in the appropriate layer by drainage from the bottom of tube or through suction. These mitochondria, now in 1.35M sucrose, were diluted slowly at 4°C with 10mM phosphate buffer containing 0.1% BSA until a sucrose concentration of about 0.3M was achieved. The time required for the dilution process was 20 to 30 min. The diluted mitochondria were centrifuged at 37,000g for 10 min and the purified mitochondria were collected and suspended in 0.2M sucrose to give a total volume of 2 ml. All operations were carried out between 4°C and 6°C and at pH 7.2. This extraction procedure is schematically illustrated in Figure 2.

A comparison between the purified and the washed mitochondria was made on the basis of their rates of oxidation of succinate, respiratory control ratio (RCR), ADP/O ratio and the total protein obtained. This was done using both 'Lodi' and 'Delicious' apples and the results are shown in Table 1. The purified mitochondria had a higher rate of oxidation than the washed mitochondria, but the quantity of protein obtained was greatly reduced. Furthermore, the RCR and ADP/O ratio were very similar which indicated that their degree of intactness was not much different. Since the quality of mitochondria appeared to be marginally

TISSUE GRATED INTO ISOLATION MEDIUM,
STRAINED THROUGH 4 LAYERS OF CHEESECLOTH

DISCARD PULP

FILTRATE
Strained through nylon fabric
(50u mesh)

DISCARD PRECIPITATE

FILTRATE
Centrifuge: 37,000g for 10 min

DISCARD SUPERNATANT

Suspend pellet in wash medium
Centrifuge: 29,000 rpm for 1½ hr
in sucrose density gradient

DISCARD the non-mitochondrial
layers

Remove mitochondrial layer
(between 1.2 and 1.45M)
Dilute to 0.3M and then centrifuge:
37,000g for 10 min

DISCARD SUPERNATANT

MITOCHONDRIAL PELLET
Suspend in 0.2M sucrose

Figure 2. Flow-chart for isolation of purified mitochondria from apple tissue.

Table 1. Comparison of Washed and Purified Mitochondria from 'Lodi' and 'Delicious' Apples.

Reaction mixtures contained: Sucrose, 0.25M; $MgCl_2$, 5mM; KH_2PO_4-K_2HPO_4 buffer, 10mM; Tris-HCl buffer, 10mM, final pH 7.2; BSA, 3 mg/ml; yeast extract, 2 mg/ml; and substrate as indicated. Apples were at preclimacteric stage of development. All values are for State IV oxidation.

	Washed Mitochondria	Purified Mitochondria
'Lodi' Apples		
Succinate Oxidation (nmole O_2/min-mg protein)	69.9	79.0
Respiratory Control Ratio	2.1	2.0
Protein Obtained (mg/ml)	15.6	3.7
'Delicious' Apples		
Succinate Oxidation (nmole O_2/min-mg protein)	112.0	162.0
Respiratory Control Ratio	1.3	1.1
ADP/O Ratio	1.0	1.1
Protein Obtained (mg/ml)	10.0	4.0

improved by purification, while the quantity of protein recovered was markedly reduced and the time for extraction was greatly increased (3 to 3½ hr) by purification, the purification procedure was abandoned. All subsequent data in this study were obtained with mitochondria extracted by the procedure of Shipway (56).

3. Determinations of Apple Mitochondria.

A. Conditions for Mitochondrial Determinations.

Most of the mitochondrial determinations were measured polarographically at 25°C using a recording oxygen cathode (Oxygraph Model KM, Gilson Medical Electronics) fitted with a Clarke-type oxygen electrode (Yellow Springs Instrument Co.) and teflon (0.001") membrane in a 1.7 ml cuvette. Polarizing voltage was maintained at 0.8v throughout. Materials were introduced into the chamber through a movable well with a small opening, and the reaction mixture was stirred by means of a small magnetic stirrer. The temperature of the reaction medium was maintained using a thermostatically controlled pressurized water jacket. The reaction medium contained: sucrose, 0.25M; $MgCl_2$, 5mM; KH_2PO_4-K_2HPO_4 buffer, 10mM; Tris-HCl buffer, 10mM; (final pH 7.2); substrates malate, pyruvate, succinate all 16mM; and NADH, 1mM. Also BSA, 3 mg/ml, and yeast extract, 2 mg/ml, were added immediately prior to assay. The latter was used as a source of co-factors.

B. Measurements of Rate of Oxidation, RCR and ADP/O Ratio.

The reaction medium was placed in the cuvette followed by substrate and 0.05 umole of ATP, and a base-line was obtained. An appropriate amount (0.05 to 0.1 ml) of mitochondrial suspension was added to initiate the experiment. Once a steady base rate was established 0.05 umole of ADP was introduced. The oxidation was stimulated (State III), and later it returned to the non-stimulated rate (State IV). With another addition of ADP the stimulated State III and the non-stimulated State IV cycled again as shown in Figure 3. This cycling of State III and State IV is one of the criteria for intact mitochondria (13). The rate of oxidation was obtained at the non-stimulated State IV after the second addition of ADP, and was calculated from a record trace on the basis of 0.25 umole O_2/ml in an air saturated medium, employing Shipway's method (56). Oxygen consumption was expressed as nmole O_2/min-mg protein. RCR was obtained as the rate of respiration in the presence of ADP divided by the rate when it had been exhausted, i.e. State III/State IV. The ADP/O ratio is the ratio between the O_2 uptake and the esterification of P_i to ADP, and was calculated as the amount of ADP added divided by the amount of oxidation at State III.

C. Effect of TPP, Arsenite and Glutamate.

The general procedure was the same as described above.

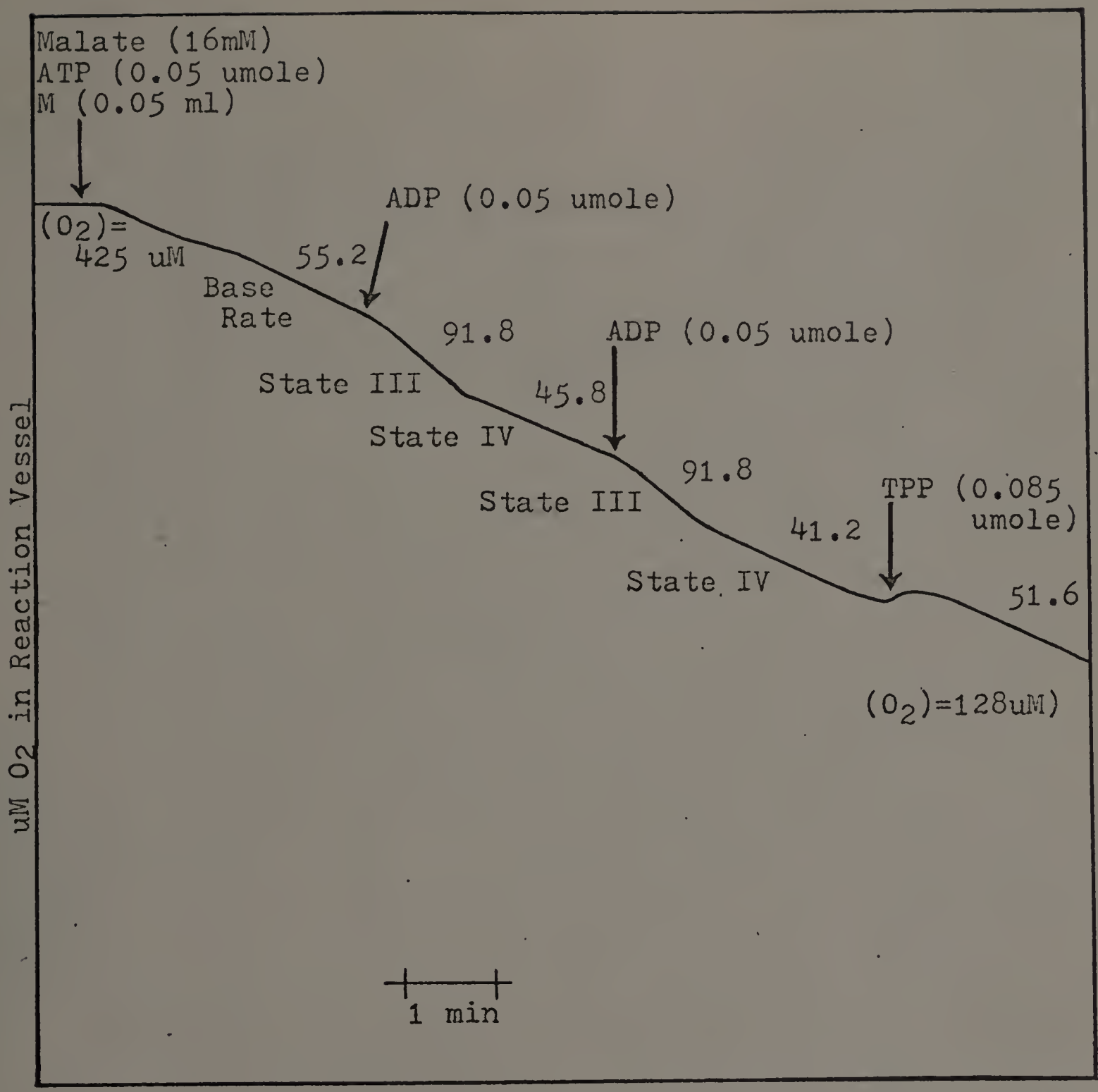

Figure 3. Polarograph trace showing the cycling of State III and State IV for malate oxidation. Assayed in base medium described in Materials and Methods. The numbers on the trace represent the uptake of O_2 in nmole O_2/min per 0.05 ml of mitochondrial suspension (M).

TPP (0.085 umole) was added at the end of the non-stimulated State IV after the second addition of ADP and the rate of oxidation was calculated as above. Sodium arsenite ($NaAsO_2$) (5.1 umoles) was then added and the steady-state rate in its presence was determined. Finally, glutamic acid (5.1 umoles) was added and when a steady rate was established the oxidation rate was determined.

D. Swelling and Contraction.

The mitochondrial swelling and contraction were measured spectrophotometically at 520 nm using the method of Earnshaw and Truelove (14) with some modification. For swelling, 0.1 ml of mitochondrial suspension (0.8-1.8 mg protein) was added to 2.9 ml of 0.1M KCl, 0.02M Tris-HCl buffer, pH 7.5. As the mitochondria swelled there was a decrease in Optical Density (O.D.). The initial reading of O.D. was taken after 1 min (zero time) and then at every min for the first 5 min. Thereafter O.D. was read at every 10 min until it reached a constant minimum value. The mitochondrial contraction was initiated by adding 8.5 umoles ATP and 5.1 umoles $MgCl_2$ after the constant maximum value for swelling was obtained, which usually required about 30-40 min. As the mitochondria contracted the O.D. increased, and O.D. was recorded in the same manner as during swelling, but the maximum value ususlly required a longer time to be obtained (1 to $1\frac{1}{2}$ hr). Typical results

of an experiment are shown in Figure 4. For simplicity in presenting experimental results, the capacities for mitochondrial swelling and contraction were expressed as O.D. decrease X 1000 and O.D. increase X 1000, respectively, after the first 5 min and at their maximum values. The reading after 5 min is presented as an indication of rate of swelling, whereas the maximum value indicates the full potential for swelling.

4. Respiration of Whole Fruit.

Whole fruit respiration was assessed by CO_2 analysis of constant-flow effluent air with a MSA model 200 infrared gas analyzer. About 1 kg of apples was used and sealed in a 9 liter desiccator at 21°C, and a continuous flow of air (10 liters/min) was monitored for CO_2 content.

5. Protein Determination.

Protein content of the mitochondrial preparation was used as an indication of the amount of mitochondria present. The Lowry method (44) of protein determination as modified by Shipway (56) was found to be reliable and consistent and thus was employed in all experiments dealing with mitochondria.

6. Organic Chemicals Used in These Studies.

Compound	Grade	Source
L-cysteine HCl	-	Schwarz/Mann
Serum albumin (bovine)	Fatty acid poor	" "

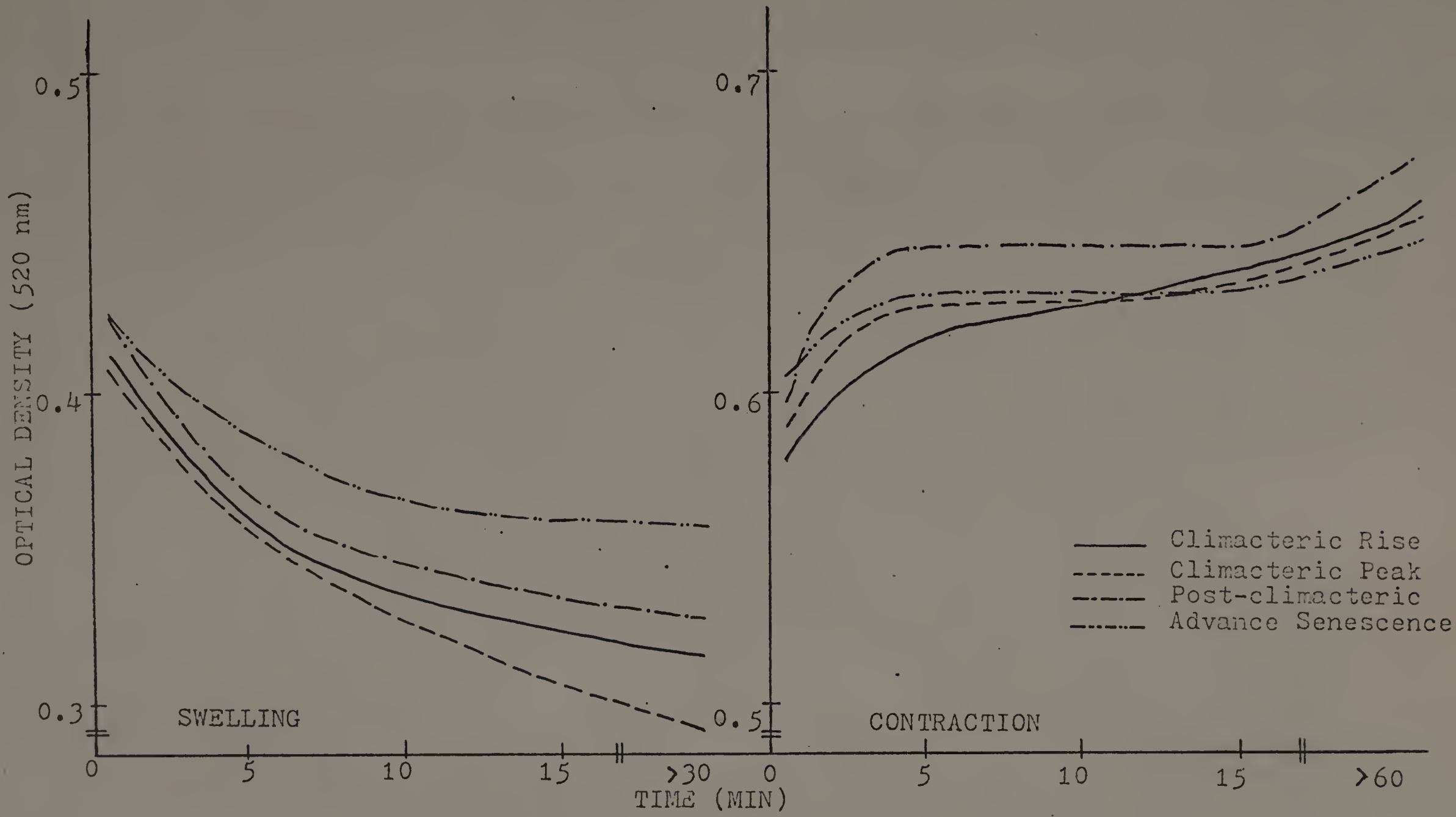

Figure 4. Swelling and contraction of mitochondria from 'Delicious' apples at different stages of development. Swelling occurred in 0.1M KCl, 0.2M Tris-HCl, pH 7.5. at minimum O.D., contraction was initiated by addition of 8.5 umoles ATP and 5.1 umole $MgCl_2$.

Compound	Grade	Source
NADH	-	Schwarz/Mann
ATP di-sodium salt	-	" "
TPP	-	" "
PVP-40	Pharma-ceutical, MW 40,000	Sigma
Succinate	-	"
Cytochrome c	Horse heart, type II-A	"
L-Glutamate mono-potassium salt		"
L-Malate	-	Nutritional Biochem. Corp.
Pyruvate	99% high purity	" " "
ADP (sodium dihydrate)	-	" " "
Yeast extract	Bio. Cert.	Fisher Scientific Co.
Tris	Cert. primary standard	" " "
Sucrose	-	Malinckrodt Chem. Works
EDTA (di-sodium salt)	-	" " "

R E S U L T S

1. Properties of Extracted Mitochondria.

Mitochondria extracted in these studies readily oxidized succinate, malate, pyruvate and NADH. With pyruvate, much better oxidation was obtained by addition of a catalytic concentration (1.6mM) of "sparker" malate to the reaction medium, as is widely recognized (1,3,41). Respiratory control was obtained with all substrates. It was observed for all traces that subsequent addition of ADP after a display of RC always gave RC ratio which was higher than after the first addition. It has been suggested that the initial introduction of ADP results in an improvement in the structure of the mitochondrial membrane leading to tighter coupling of oxidation and phosphorylation (36).

Preliminary work showed that addition of ATP at the beginning of the assay gave a slightly higher RC ratio; furthermore, Douce et al. (13) showed that such addition of ATP removed the inhibition by product accumulation in malate oxidation, i.e. the inhibited State IV. Therefore, 0.05 umole ATP was added at the beginning of each assay, to assure better oxidation. Also, 0.38 umole of cytochrome c was added for each set of assays, under the base conditions since cytochrome c may be lost from mitochondria if damage occurs to mitochondria during extraction. Stimulat-

ion from this addition was never seen.

2. Activity of Mitochondria at Different Stages of Fruit Ripening.

A. Comparison of Whole Fruit Respiration and Mitochondrial Oxidations.

Production of CO_2 by intact fruit showed that the 4 lots of fruit designated "Climacteric Rise", "Climacteric Peak", "Post-climacteric", and "Advanced Senescence" exhibited in composite a typical climacteric respiration pattern (Table 2). These values are for respiration after 1 day at 21°C, to allow temperature equilibration, and were followed by changes typical of fruit at these stages of development.

Mitochondrial oxidations of succinate, malate, pyruvate, and NADH were determined at each stage of ripening (Table 2). With all substrates the oxidation rate appeared to be higher at the climacteric peak than at the climacteric rise, but differences were statistically significant only with pyruvate as substrate. The slight fall in whole fruit respiration at the post-climacteric stage was not accompanied by any significant reduction of mitochondrial oxidation. However, with advanced senescence, mitochondrial oxidations of all substrates were substantially reduced, in accompaniment with a sizeable reduction of whole fruit respiration. It seems likely that with further replication

Table 2. Comparison of Whole Fruit Respiration and Mitochondrial Oxidations of Succinate, Malate, Pyruvate and NADH at Different Stages of Ripening of 'Delicious' Apples.

Reaction mixtures contained: Sucrose, 0.25M; $MgCl_2$, 5mM; KH_2PO_4-K_2HPO_4 buffer, 10mM; Tris-HCl buffer, 10mM, final pH 7.2; BSA 3 mg/ml; yeast extract 2 mg/ml; and substrate as indicated. All values are for State IV oxidation and are averages of 3 replications, with standard deviation of the mean indicated.

Stage of Ripening	Whole Fruit Respiration	Total Protein Extracted	Substrate			
			Succinate	Malate	Pyruvate	NADH
	mg CO_2/kg-hr		nmole O_2/min-mg protein			
Climacteric Rise	19.8	15.0±1.0	148.8±13.0	43.4±13.2	19.5±3.2	347.4± 57.5
Climacteric Peak	26.9	13.3±0.6	172.2±36.3	53.7± 6.8	35.7±2.5	430.7±136.6
Post-climacteric	23.3	14.2±0.8	162.8±17.0	55.1± 7.4	36.3±2.1	345.3± 78.9
Advanced Senescence	20.2	14.6±0.3	98.7±17.7	45.2± 2.7	19.1±2.9	183.5± 32.3

greater significance for mitochondrial oxidation rates would have been recorded for all substrates as the fruit went from the climacteric rise to the climacteric peak. Nevertheless, if a large number of replications are necessary to identify such an increase, the change would not seem to be of great consequence to the fruit. However, the markedly lower mitochondrial oxidations associated with the lowered whole fruit respiration at advanced senescence suggest a close relationship between these 2 phenomena at this stage of development. These results suggest that reduced mitochondrial oxidative capacity may be integrally associated with the respiratory decline with advanced senescence, but that other factors may play more significant roles in the respiratory rise during the climacteric.

B. Respiratory Control and ADP/O Ratios during Fruit Ripening.

To measure the capacity of mitochondria to generate ATP, 0.05 umole of ADP was added to the reaction medium during each determination (Table 2) under base condition (oxidation rate prior to ADP addition), and the stimulated State III and restored State IV conditions were recorded. From the recorded traces, both the Respiratory Control Ratio (RCR, State III/State IV) and the ratio of ADP utilization to O_2 consumption (ADP/O ratio) during the period were calculated as measures of the degree of coupl-

ing of phosphorylation to oxidation. Although these ratios (Table 3) are not as high as reported for some other tissues (31, 13), they are equivalent to those previously reported for apple mitochondria (29, 60).

There was no change in either RCR or ADP/O ratio for mitochondria on any substrate during fruit ripening (Table 3). Even with advanced senescence the phosphorylative capacity of the mitochondria was unchanged. Thus, neither the rise in whole fruit respiration to the climacteric peak nor the fall during advanced senescence can be attributed to changes in the phosphorylative capacity of mitochondria.

Theoretically, the ADP/O ratio for malate and pyruvate should be 3.0, while that for succinate should be 2.0, although recorded values for plant mitochondria are usually considerably lower (30). The lower ADP/O ratio of succinate than of malate or pyruvate (Table 3) is thus expected. The somewhat lower value of NADH is also excepted, for although the theoretical value for endogenous NADH is 3.0 in animal mitochondria, the maximum value for exogenous NADH using plant mitochondria is 2.0 due to the presence of a second, cyanide-insensitive cytochrome pathway for its oxidation (30). RC ratios of infinity are possible with complete ADP dependence and absence of ATPase, but even highly purified plant mito-

Table 3. Respiratory Control and ADP/O Ratios on Succinate, Malate, Pyruvate and NADH exhibited by Mitochondria from 'Delicious' Apples at Different Stages of Ripening.

Experimental conditions as for Table 2.

Stage of Ripening	Substrate			
	Succinate	Malate	Pyruvate	NADH
Respiratory Control Ratio				
Climacteric Rise	1.3±0.1	1.5±0.2	1.4±0.2	1.3±0.1
Climacteric Peak	1.4±0.1	1.8±0.5	1.2±0.1	1.2±0.0
Post-climacteric	1.4±0.1	1.8±0.4	1.2±0.2	1.3±0.1
Advanced Senescence	1.5±0.2	1.8±0.4	1.4±0.1	1.4±0.0
ADP/O Ratio				
Climacteric Rise	1.1±0.1	1.7±0.7	1.7±0.4	1.2±0.1
Climacteric Peak	1.0±0.1	1.5±0.5	1.5±0.1	1.2±0.1
Post-climacteric	1.0±0.0	1.4±0.1	1.6±0.3	1.2±0.1
Advanced Senescence	1.1±0.1	1.6±0.3	1.7±0.4	1.3±0.2

chondria exhibited RC ratios of no more than 2.0 to 5.5, depending on substrate (13). For apple mitochondria, the highest reported RC ratios are 2.0 to 2.8 (56).

C. Effect of TPP on Mitochondrial Oxidation of Various Substrates during Fruit Ripening.

Since a stimulation of fruit mitochondria by TPP during the climacteric has been reported (36) and considerable significance has been attributed to it (37), 0.085 umole of TPP was added under State IV conditions to mitochondria on various substrates. TPP stimulation of succinate or NADH oxidation would not be expected, since it is not a co-factor for these reactions. Non-replicated additions of TPP at all stages of ripening confirmed that it did not stimulate oxidation of succinate or NADH, although it may have reduced NADH oxidation (Table 4). TPP is a recognized co-factor for pyruvate oxidation, and its addition to the base medium stimulated pyruvate oxidation; a pronounced stimulation resulted at both the climacteric rise and climacteric peak, but even at advanced senescence a significant stimulation occurred. With malate, TPP appeared to have an effect at the climacteric peak and post-climacteric periods but the differences were not statistically significant. Any effect on malate is likely the indirect reflection of stimulated pyruvate oxidation. These results from TPP additions resemble the results of

Table 4. Effects of TPP on Oxidations of Succinate, Malate, Pyruvate and NADH by Mitochondria from 'Delicious' Apples at Different Stages of Ripening.

Experimental conditions as for Table 2 except that 0.085 umole of TPP was added after the second addition of ADP following the State IV for all +TPP values. All -TPP values are for State IV oxidation.

Stage of Ripening	Substrate							
	Pyruvate		Malate		Succinate		NADH	
	-TPP	+TPP	-TPP	+TPP	-TPP	+TPP	-TPP	+TPP
	nmole O_2/min-mg protein							
Climacteric Rise	19.5±3.2	40.3±11.8	43.4±13.2	45.7± 5.3	132.1	127.6	676.1	640.4
Climacteric Peak	35.7±2.5	55.4± 6.8	53.7± 6.8	63.7±10.9	179.8	173.6	---	---
Post-climacteric	36.3±2.1	37.9± 0.9	55.2± 7.4	62.7± 9.8	159.6	156.8	625.5	469.8
Advanced Senescence	19.1±2.9	25.1± 1.4	45.2± 2.7	46.6± 8.5	98.0	94.4	373.1	348.1

Hobson (22) using tomato mitochondria, but do not agree with the findings of Lance *et al.* (37) with avocados or of Hulme *et al.* (29) using apples. These findings are supported by the results of a preliminary experiment using 'Lodi' apples (Appendix Table 2) in which mitochondria from fruit passing through the climacteric were tested for TPP stimulation. Pyruvate oxidation at all stages appeared to be stimulated by TPP. There appears to be a relatively constant stimulation of pyruvate oxidation by exogenous TPP throughout the respiratory climacteric of apples.

D. Capacity of Mitochondria for Swelling and Contraction During Fruit Ripening.

Differences existed in the swelling and contraction of mitochondria extracted from fruit of different developmental stages (Table 5). Maximum swelling was greater for mitochondria at the climacteric peak and post-climacteric than at either the climacteric rise or advanced senescence. However, the extent of swelling after 5 min, which is taken as an indication of rate of swelling, was similar for mitochondria during the climacteric until advanced senescence; at this point it was substantially reduced, as was the maximum swelling.

Mitochondrial contraction was considerably more variable than the preceding swelling, and the only signi-

Table 5. Capacity for Swelling and Contraction by Mitochondria from 'Delicious' Apples at Different Stages of Ripening.

For swelling 0.1 ml of mitochondrial suspension was added to 2.9 ml of 0.1M KCl, 0.02M Tris-HCl buffer, pH 7.5 and O.D. at 520 nm was followed. For contraction 8.5 umoles ATP and 5.1 umoles $MgCl_2$ were add after the maximum value of swelling and O.D. was followed. Results are averages of 3 replications, with standard deviation of the mean indicated.

Stage of Ripening	Swelling		Contraction	
	1st 5 min	Maximum	1st 5 min	Maximum
	O.D. decrease X 1000		O.D. increase X 1000	
Climacteric Rise	24.8±6.7	49.4±4.2	30.2±2.5	63.9±15.6
Climacteric Peak	26.8±5.4	65.0±9.2	28.9±4.7	66.8±20.6
Post-climacteric	34.2±3.1	61.6±3.6	30.6±6.0	49.4± 4.6
Advanced Senescence	18.8±0.7	30.4±2.9	26.6±6.3	31.3± 3.2

ficant difference existed at advanced senescence, at which time, maximum contraction was greatly reduced (Table 5). It is interesting to note that mitochondria from these senescent fruit achieved nearly complete contraction in 5 min, while those from less senescent fruit were only about 50% contracted at this time.

Since capacity for swelling and contraction can be a reflection of membrane composition and flexibility, these data may indicate that mitochondrial membrane changes occur during ripening of apples. Increased flexibility may accompany the climacteric rise, but it would appear that the change is not substantial. It seems clear that a considerable decrease in flexibility does accompany advanced senescence, as both swelling and contraction were sharply reduced at this time.

3. Mitochondrial Activity of Fruit during Ripening at 21°C.

The preceding experiments were with mitochondria from lots of fruit maintained in cold storage at specific stages of ripeness. It was of interest to follow changes in a single lot of fruit during its ripening. A small preliminary experiment of this type had been conducted earlier with 'Lodi' apples (Appendix Tables 1 and 2).

A lot of 'Richared Delicious' apples that had been harvested at the preclimacteric stage was obtained from controlled atmosphere storage in January, 1973. These fruit

were transferred to 21°C air and changes were followed for 16 days. Unfortunately, these fruit did not undergo the normal respiratory climacteric during their ripening, as demonstrated by the whole fruit respiratory pattern during this time (Table 6). The fruit underwent ripening changes (color, texture, aroma etc.) during this time, and the lack of the characteristic climacteric was presumably a carryover effect of the storage conditions. Because a climacteric did not occur, the experiment was not repeated and results cannot be used to help interpret the climacteric. However, the data help characterize the behavior of fruit mitochondria and are presented below.

A. Comparison of Whole Fruit Respiration and Mitochondrial Oxidations of Various Substrates.

Whole fruit respiration was almost constant for 11 days, after which it appeared to decline (Table 6). Mitochondrial oxidations of succinate, malate, and pyruvate were generally uniform throughout this period, except that NADH oxidation seemed to be higher early in the experiment. It is noteworthy that neither whole fruit nor mitochondria exhibited the rise in activity recorded earlier (Table 2), which suggests that the presumed carryover effect of controlled atmosphere storage on respiration may be exerted at the mitochondrial level.

Table 6. Comparison of Whole Fruit Respiration and Mitochondrial Oxidations of Succinate, Malate, Pyruvate and NADH by Mitochondria from 'Delicious' Apples kept for Different Intervals of 21°C.

Experimental conditions as Table 2.

Time at 21°C	Whole Fruit Respiration	Substrate			
		Succinate	Malate	Pyruvate	NADH
days	mg CO_2/kg-hr	nmole O_2/min-mg protein			
0	---	127.23	29.74	15.75	167.27
1	29.6	110.32	49.50	16.41	191.41
2	29.6	117.36	41.17	22.89	205.84
3	29.9	109.49	33.15	19.60	172.17
7	31.4	101.97	34.90	22.30	133.36
8	30.5	94.64	42.30	16.24	114.01*
11	30.5	96.87	43.29	19.75	141.50
14	25.1	93.21	46.60	15.97	112.38*
16	24.8	104.10	53.99	19.11	113.89*

* Amount of mitochondria used was twice that of other determinations.

B. Respiratory Control Ratio and ADP/O Ratio on Various Substrates.

As in the previous experiment (Table 3), RC and ADP/O ratios were relatively uniform during the ripening of fruit (Table 7). Furthermore, except for the RC ratio with NADH, the values of these ratios were comparable to those in the earlier experiment. The RC ratio with NADH appeared to somewhat higher in this latter experiment.

C. Capacity of Mitochondria to Swell and Contract.

Throughout the experiment, no distinct change in mitochondrial capacity for swelling or contracting was evident (Table 8). There was an indication of slightly greater rate of and capacity for swelling later in the experiment, but if real this had no relation to whole fruit respiration or mitochondrial oxidations (Table 6), or to observed ripening changes.

The most striking feature of swelling and contraction of mitochondria from these fruit is the considerably lower values here than in the earlier experiment (Table 5). This perhaps reflects an effect of controlled atmoshpere storage on the fruit, and possibly accounts in part for their failure to express the typical respiratory climacteric.

D. Effects of TPP, Arsenite, and Glutamate on Mitochondrial Oxidations of Various Substrates.

Table 7. Respiratory Control and ADP/O Ratios on Succinate, Malate, Pyruvate and NADH exhibited by Mitochondria from 'Delicious' Apples held at Different Intervals of 21°C.

Experimental conditions as in Table 3.

Time at 21°C	Substrate			
	Succinate	Malate	Pyruvate	NADH
days				
Respiratory Control Ratio				
0	1.4	1.4	1.2	1.7
1	1.4	1.7	1.1	1.5
2	1.4	1.6	1.3	1.5
3	1.3	1.9	1.2	1.3
7	1.6	2.0	1.3	1.8
8	1.4	1.7	1.5	1.8
11	1.4	1.8	1.3	1.7
14	1.8	1.8	1.9	1.8
16	1.6	2.1	1.6	2.0
ADP/O Ratio				
0	1.1	1.5	1.6	1.2
1	0.9	1.3	2.4	1.5
2	0.9	1.5	1.8	1.2
3	1.1	1.3	1.6	1.2
7	1.2	1.5	2.6	1.8
8	1.3	1.5	2.6	1.3
11	1.1	1.8	2.1	1.8
14	1.1	1.2	1.2	1.3
16	1.2	1.5	1.5	1.5

Table 8. Capacity for Swelling and Contraction by Mitochondria from 'Delicious' Apples Held for Different Intervals at 21°C.

Experimental conditions as in Table 5.

Time at 21°C	Swelling		Contraction	
	1st 5 min	Maximum	1st 5 min	Maximum
days	O.D. decrease X 1000		O.D. increase X 1000	
0	9.8	19.1	---	---
1	16.8	22.1	13.0	25.1
2	16.3	18.3	7.0	25.7
3	14.4	18.0	10.1	28.1
7	16.4	24.2	11.1	27.2
8	17.2	23.6	13.8	31.6
11	22.4	24.1	13.8	22.4
14	20.3	25.5	15.0	23.1
16	20.9	26.2	---	---

Previous data (Tables 6 to 8) indicate that mitochondrial properties changed little during 16 days at 21°C. At 9 intervals during this time, the effects of sequential additions of TPP, arsenite (AsO_2^-), and glutamate were determined during oxidation of pyruvate, malate, succinate, and NADH. The purpose of this experiment was to determine if responses reported by Lance et al. (37) for mitochondria from ripening avocados could be reproduced using apple mitochondria. The mitochondrial responses to these additives were remarkably uniform during the 16 days at 21°C, with the possible exception that AsO_2^- inhibition seemed to be slightly greater later in the experiment (Appendix Tables 3 to 6). Since mitochondrial properties changed little and responses to additives were generally uniform during the experiment, responses to additives have been averaged and standard errors have been calculated for the 9 determinations (Table 9).

The addition of 0.085 umole of TPP to the reaction medium doubled the rate of pyruvate oxidation, but had no distinguishable effect on malate, succinate or NADH oxidations. These results support the earlier findings (Table 4) with pyruvate and succinate, and suggest that the small (and statistically insignificant) stimulation of malate oxidation seen earlier was not real.

Arsenite, an inhibitor of the pyruvate oxidase complex

Table 9. Effects on Mitochondrial Oxidations of Various Substrates from Sequential Additions of TPP, Arsenite, and Glutamate.

Base reaction medium as in Table 2. Basal rate are for State IV oxidation. Additions of 0.085 umole of TPP, 5.1 umoles of AsO_2^-, and 5.1 umoles of glutamate were made at steady state following the preceding addition. Data are steady-state averages of 9 determinations during 16 days at 21°C, using 'Richared Delicious' apples taken from controlled atmosphere storage.

Substrate	Additive			
	Basal	TPP	AsO_2^-	Glutamate
	nmole O_2/min-mg protein			
Pyruvate	18.7±2.5	37.6± 5.9	8.3± 3.1	24.6± 6.0
Malate	41.6±8.1	40.9± 8.3	25.8± 5.8	23.2± 4.4
Succinate	106.1±11.6	103.6±12.8	77.5±16.6	70.5±18.0
NADH	150.2±35.3	118.5±31.3	52.7±10.9	57.5±14.4

at the α-lipoic acid level (37, 39), suppressed pyruvate oxidation markedly but not completely (Table 9). It also reduced oxidation of malate, succinate and NADH, but to a considerably lesser extent than pyruvate. According to Lance et al. (37), AsO_2^- inhibition of pyruvate oxidation results in accumulation of oxaloacetate, which inhibits further TCA oxidations by the mitochondria. In their work, AsO_2^- inhibited malate oxidation by avocado mitochondria, presumably via accumulation of oxaloacetate (OAA), since this inhibition was largely relieved by the addition of glutamate, which undergoes transamination with OAA to yield aspartate and α-ketoglutarate. The addition of glutamate to apple mitochondria largely relieved AsO_2^- inhibition of pyruvate oxidation, but had no effect on its inhibition of malate, succinate or NADH oxidations (Table 9). Thus, our findings do not agree with those of Lance et al. (37) and suggest that mitochondrial systems of ripening avocados and apples function differently.

D I S C U S S I O N

Among those who have studied mitochondrial activity during fruit ripening, 2 divergent viewpoints exist. On one hand there is the frequently cited conclusion of Hulme *et al.* (25) that mitochondrial activity increases during the climacteric of apples, while on the other hand there are the careful investigations of Lance *et al.* (36, 37) that led to the conclusion that mitochondrial activity *per se* does not increase during the climacteric of avocados. Each argument can be supported by studies of others.

Interpretation of the present study is hampered by lack of known preclimacteric fruit except in the preliminary study with 'Lodi' apples. However, the data support the conclusion of Hulme *et al.* (25) that activity *per se* is greatest at the climacteric peak (Table 2). The significance of such an increase within the fruit is questionable, however. The increase in activity at the climacteric peak was relatively small and variable to account for the respiratory rise in the whole fruit, during the climacteric. Also, the increase in respiration was observed under *in vitro* condition in which the mitochondria are presumably functioning at their maximum rate. This maximum activity may be limited *in vivo* by many factors such as the availability of substrate or of co-factors in the mitochondria. It cannot be assumed that because *in vitro* activity in-

creases during this period, this increase is being expressed within the intact tissue.

Perhaps, more significant than the relationship between mitochondrial activity and the climacteric rise is the relationship between the subsequent decline in mitochondrial activity and whole fruit respiration with advanced senescence. Both declines were sizeable (Table 2). The in vitro decline might be attributed to mitochondrial disintegration during senescence. Electron microscopic examination of mitochondria from ripening pears has shown that these organelles remain discrete and distinguishable well after the rest of the cell has undergone massive deterioration (2), yet it is possible that subtle changes have occurred that inhibit their oxidative capacity. The possibility that mitochondrial disruption occurs during this time was suggested by the findings of Jones et al. (33) that the ADP/O ratio of mitochondria fell as apples became "overripe". Using α-ketoglutarate as substrate, they found the ADP/O ratio to fall from 1.8 to 0.52. Our findings conflict with these; neither the ADP/O nor the RCR fell when 'Delicious' apples were "overripe" (Advanced Senescence, Table 3). Likewise, the ADP/O ratio with ripening avocados was found not to fall (36), and Australian workers reported that apples retained a high oxidative phosphorylative ability for 4 to 12 months in $0^{o}C$ air

storage after the climacteric rise (41).

The capacity of mitochondrial swelling and contraction is interpreted to reflect the integrity and flexibility of the mitochondrial membranes. It is therefore interesting to observe the sharp decline in swelling and contraction with advanced senescence (Table 5). Although Bain and Mercer (2) found that mitochondria are among the last cellular organelles to disintegrate during fruit senescence, the electron microscopic studies by Stoner and Hanson (58) showed that the mechanism of mitochondrial swelling and contraction is associated with the inner membrane. During contraction the mitochondria showed dense cristae or involutions of the inner membranes and during swelling the mitochondria appeared to have an inner membrane which was partially plasmolyzed while the less dense outer membrane seemed unaffected. In our study, mitochondrial swelling and contraction were sharply reduced at advanced senescence. This may indicate that changes had occurred in the inner membranes of the mitochondria, which in turn might account for reduced oxidative capacity.

It is also interesting to note the significantly greater swelling at the climacteric peak than during the climacteric rise. This suggests that some alteration of mitochondrial properties occurs during this time, which may be more important than mitochondrial oxidation rates

per se; e.g., permeability to substrates may be greater. The greatly reduced swelling and contraction of mitochondria from apples after controlled atmosphere storage is also noteworthy. Perhaps, the suppression of mitochondrial oxidations during controlled atmosphere storage observed by Shipway (56) is attributable to this effect. Additional research into mitochondrial swelling and contraction during fruit storage and ripening is warranted.

Lance et al. (37) concluded that TPP availability may be a controlling factor in the respiratory rise, since avocado mitochondria oxidizing malate were stimulated by exogenous TPP at the preclimacteric but not at the climacteric peak. This view was not supported by Hobson's study with tomatoes (22). TPP is an absolute requirement for oxidation of pyruvate to acetyl CoA, as well as for the oxidation of α-ketoglutarate to succinyl-CoA. TPP addition did not significantly stimulate the oxidation of succinate and malate (Table 4 and 9), probably due to our inclusion of yeast extract in the base reaction medium, which likely provided sufficient TPP to maintain TCA operation with these substrates. With pyruvate as substrate, TPP is required at two sites if the TCA cycle is operating, and its addition stimulated oxidation. However, this stimulation was consistent throughout ripening (Table 4) and after controlled atmosphere storage (Table 9). These results,

along with those of Hobson (22), argue against a controlling role for TPP availability during the respiratory climacteric, since responses to exogenous additions did not change during this period.

Another factor given importance by Lance et al. (37) was the avoidance of OAA accumulation within the TCA cycle. Their data suggested that in avocado mitochondrial preparations a large proportion of malate is converted to pyruvate and then condensed with OAA to form citrate, thus avoiding inhibitory accumulation of OAA. This process was enhanced by added TPP and inhibited by arsenite (AsO_2^-), with the inhibition being relieved by added glutamate. Our apple mitochondrial preparations did not respond in this manner. TPP did not stimulate malate oxidation, and while AsO_2^- partially inhibited its oxidation, the inhibition was not overcome by glutamate (Table 9). Furthermore, the AsO_2^- inhibition was no greater than that where succinate or NADH served as substrate. The failure of glutamate to relieve malate inhibition may be due to the AsO_2^- inhibition of the oxidation of α-ketoglutarate, which is one of the products of the OAA-glutamate transamination. It does not appear that OAA accumulation and its removal are playing controlling roles in oxidation by apple mitochondria.

This investigation supports the contention that

mitochondrial changes occur during the rise and fall of the respiratory climacteric. However, these changes may contribute more to the respiratory fall than to its preceding rise. Furthermore, these changes may be accounted for more by changes in mitochondrial physical properties, such as swelling and contraction, than by changes in rates of enzymatic reactions. TPP, AsO_2^- and glutamate had marked effects on pyruvate oxidation but not on oxidations of malate, succinate and NADH. Therefore, TPP does not appear to be the controlling factor for the changes in respiration during the climacteric, as proposed by Lance et al. (37).

S U M M A R Y

Experiments were conducted to determine whether the increase in respiration during the climacteric in 'Delicious' apples is related to changes in mitochondria. 'Delicious' apples at different climacteric stages were used for the determinations of whole fruit respiration, mitochondrial oxidations, and RC and ADP/O ratios, on various substrates. Effects of TPP, arsenite and glutamate were examined, and mitochondrial swelling and contraction were determined.

The results are summarized as follows:

1. The extracted mitochondria were capable of oxidizing succinate, malate pyruvate and NADH.
2. Pyruvate oxidation was higher at the climacteric peak than during the climacteric rise; while there was an indication of increased activity with the other substrates also, the differences were not significant. However, significant decreases were found in oxidations of all substrates at an advanced senescence stage.
3. Neither RC nor ADP/O ratio changed during the climacteric or senescence.
4. TPP stimulated pyruvate oxidation at the climacteric rise, climacteric peak and at advanced senescence. No significant stimulatory effect was found for malate,

succinate or NADH oxidation at any stage of development.

5. In the presence of TPP, arsenite had inhibitory effect on mitochondrial oxidation on succinate, malate, NADH and especially on pyruvate.

6. After the successive additions of TPP, arsenite and glutamate, glutamate relieved arsenite inhibition of pyruvate oxidation, but not that of malate, succinate, or NADH.

7. The capacity for mitochondrial swelling increased at the climacteric peak and decreased at advanced senescence. The capacity for mitochondrial contraction also decreased markedly at advanced senescence.

8. Apples from controlled atmosphere storage exhibited no respiratory climacteric in the whole fruit respiration and also in the oxidations of succinate, malate, pyruvate and NADH. Furthermore, these fruit underwent less swelling and contraction than air-stored fruit.

L I T E R A T U R E C I T E D

1. Avron, A. M. and J. B. Biale. 1957. Metabolic process in cytoplasmic particles of avocado fruit. III. The operation of the tricarboxylic acid cycle. Plant Physiol. 32: 100-105.

2. Bain, J. M. and F. V. Mercer. 1964. Organization resistance and the respiration climacteric. Austral. J. Biol. Sci. 17: 78-85.

3. Beevers, H. and D. A. Walker. 1956. The oxidative activity of particulate fractions from germinating castor beans. Biochem. J. 62: 114-120.

4. Biale, J. B. 1960. Respiration of fruits. Encyclopedia of Plant Physiology XII (2): 536-592.

5. Biale, J. B. 1964. Growth, maturation, and senescence in fruit. Science 146: 880-888.

6. Blackmon, W. J. and D. E. Moreland. 1971. Adenosine triphosphatase activity associated with mung bean mitochondria. Plant Physiol. 47: 532-536.

7. Bonner, W. D., Jr. 1967. A general method for the preparation of plant mitochondria. In: R. E. Estabrook and M. E. Pullman, eds., Methods in Enzymology, Vol. X. Academic Press, New York. pp. 126-133.

8. Brady, C. J., J. K. Palmer, P. B. H. O'Connell and R. M. Smillie. 1970. An increase in protein synthesis during ripening of the banana fruit. Phytochem. 9: 1037-1047.

9. Burg, S. P. 1968. Ethylene, plant senescence, and abscission. Plant Physiol. 43: 1503-1511.

10. Chance, B. and G. R. Williams. 1955. A method for the localization of sites for oxidative phosphorylation. Nature 176: 250-254.

11. Chance, B. and G. R. Williams. 1956. The respiratory chain and oxidative phosphorylation. Advan. Enzymol. 17: 65-134.

12. Dickson, D. B. and J. B. Hanson. 1965. Comparison of mitochondria from tomato fruits at various stages of ripeness. Plant Physiol. 40: 161-165.

13. Douce, R., Eva L. Christenson and W. D. Bonner, Jr. 1972. Preparation of intact plant mitochondria. Biochem. et Biophys. Acta 46: 148-160.

14. Earnshaw, M. J. and B. Truelove. 1968. Swelling and contraction of Phaseolus hypocotyl mitochondria. Plant Physiol. 43: 121-129.

15. Frenkel, C., I. Klein and D. R. Dilley. 1968. Protein synthesis in relation to ripening of pome fruit. Plant Physiol. 43: 1146-1153.

16. Gerber, C. 1897. Maturation des fruits charnus. Annls. Sci. Nat. Bot., Sec. VIII tIV, p.1-277. (Original not seen, cited by Rhodes, 1970).

17. Gustafson, F. G. 1929. Growth studies on fruits, respiration of tomato fruits. Plant Physiol. 4: 349-356.

18. Haard, N. F. and H. O. Hultin. 1967. An improved technique for the isolation of mitochondria from plant tissue. Anal. Biochem. 24: 299-303.

19. Hanson, J. B., S. S. Malhotra, and C. D. Stoner. 1965. Action of calcium on corn mitochondria. Plant Physiol. 40: 1033-1040.

20. Hatch, M. D., J. A. Pearson, A. Millerd and R. N. Robertson. 1959. Oxidation of Krebs cycle acids by tissue slices and cytoplasmic particles from apple fruit. Austral. J. Biol. Sci. 12: 167-174.

21. Hedman, R. 1965. Properties of isolated skeletal muscle mitochondria from the rat. Exp. Cell. Res. 38: 1-12.

22. Hobson, G. E. 1970. The oxidation of malate by mitochondria from normal and abnormal tomato fruit. Phytochem. 9: 2257-2263.

23. Honda, S. T., and A. M. Muenster. 1960. Optically measured and packed volume of lupine mitochondria. Arch. Biochim. Biophys. 88: 118-127.

24. Hulme, A. C. 1954. Studies in the nitrogen metabolism of apple fruit. J. Exptl. Bot. 5: 159-172.

25. Hulme, A. C., J. D. Jones, and L. S. C. Wooltorton. 1963. The respiration climacteric in apple fruits. Proc. Roy. Soc. London. Series B. 158: 514-535. (Original not seen, cited by Rhodes, 1970)

26. Hulme, A. C., J. D. Jones and L. S. C. Wooltorton. 1964. Mitochondrial preparations from the fruit of the apple. I. Preparation and general activity. Phytochem. 3: 173-188.

27. Hulme, A. C., M. J. C. Rhodes. 1971. Pome fruit. In: The Biochemistry of Fruit and their Products. Vol. II. Academic Press. London, and New York. p. 333-374.

28. Hulme, A. C., M. J. C. Rhodes, T. Galliard and L. S. C. Wooltorton. 1968. Metabolic changes in excised fruit tissue. IV. Changes occurring in discs of apple peel during the development of the respiration climacteric. Plant Physiol. 43: 1154-1161.

29. Hulme, A. C., M. J. C. Rhodes, and L. S. C. Wooltorton. 1967. The respiration climacteric in apple fruit: some possible regulatory mechanism. Phytochem. 6: 1343-1351.

30. Ikuma, H. 1972. Electron transport in plant respiration. Ann. Rev. Plant Physiol. 23: 419-436.

31. Ikuma, H., and W. D. Bonner, Jr. 1967. Properties of higher plant mitochondria. I. Isolation and some characteristics of tightly-coupled mitochondria from dark-grown mung bean hypocotyls. Plant Physiol. 42: 67-75.

32. Jones, J. D., A. C. Hulme and L. S. C. Wooltorton. 1964. The respiration climacteric in apple fruits. New Phytol. 64: 158-167.

33. Jones, J. D., L. S. C. Wooltorton and A. C. Hulme. 1964. Mitochondrial preparation from the fruit of the apple II. Oxidative phosphorylation. Phytochem. 3: 201-212.

34. Kidd F. and C. West. 1922. Rep. Fd. Invest. Bd. for 1921, 14.
(Original not seen, cited by Hulme et al., 1971).

35. Lance, C. and W. D. Bonner, Jr. 1968. The respiration chain components of higher plant mitochondria. Plant Physiol. 43: 756-766.

36. Lance, C., G. E. Hobson, R. E. Young and J. B. Biale. 1965. Metabolic processes in cytoplasmic particles of the avocado fruit. VII. Oxidative and phosphorylative activities throughout the climacteric cycle. Plant Physiol. 40: 1116-1123.

37. Lance, C., G. E. Hobson, R. E. Young and J. B. Biale. 1967. Metabolic processes in cytoplasmic particles of the avocado fruit. IX. The oxidation of pyruvate and malate during the climacteric cycle. Plant Physiol. 42: 471-478.

38. Lehninger, A. L. 1964. The Mitochondria. Chapter X. Energy coupled changes of volume and structure. Benjamin, New York.

39. Lehninger, A. L. 1970. Biochemistry. Worth, Inc. New York. p. 833.

40. Leonard E. R. 1941. Studies in tropical fruits X. Preliminary observations on transpiration during ripening. Ann. Bot. 5: 89-119.

41. Lewis, T. L. and D. Martin. 1965. Protein nitrogen content and phosphorylative activity of apple fruits during ripening and senescence. Austral. J. Biol. Sci. 18: 1093-1101.

42. Lieberman, M. 1958. Isolation of cytoplasmic particles with cytochrome oxidase activity from apple. Science 127: 189-190.

43. Lieberman, M. and J. B. Biale. 1956. Co-factor requirements for the oxidation of α-keto acids by sweet potato mitochondria. Plant Physiol. 31: 425-429.

44. Lowry, D. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

45. Lyons, J. M., W. B. McGlasson and H. K. Pratt. 1962. Ethylene production, respiration, and internal gas concentrations in cantaloupe fruit at various stages of maturity. Plant Physiol. 37: 31-36.

46. Lyons, J. M. and H. K. Pratt. 1964. An effect of ethylene on swelling of isolated mitochondria. Arch. Biochem. Biophys. 104: 318-324.

47. Lyons, J. M., T. A. Wheaton and H. K. Pratt. 1964. Relationship between the physical nature of mitochondrial membranes and chilling sensitivity in plants. Plant Physiol. 39: 262-268.

48. Marks, J. D., R. Bernlohr and J. E. Varner. 1957. Esterification of phosphate in ripening fruit. Plant Physiol. 32: 259-262.

49. Millerd, A., J. Bonner and J. B. Biale. 1953. Climacteric rise in fruit respiration as controlled by phosphorylative coupling. Plant Physiol. 28: 521-531.

50. Pearson, J. A. and R. N. Robertson. 1954. The physiology of growth in apple fruits. Austral. J. Biol. Sci. 7: 1-17.

51. Rhodes, M. J. C. 1970. The climacteric and ripening of fruits. In: The Biochemistry of Fruit and their Products. Vol. I. Academic Press. London and New York. p. 521-533.

52. Rhodes, M. J. C. and L. S. C. Wooltorton. 1967. The respiration climacteric in apple fruits - the action of hydrolytic enzyme in peel tissue during the climacteric period in fruit detached from the tree. Phytochem. 6: 1-12.

53. Rhodes, M. J. C. and L. S. C. Wooltorton. 1968. A new fluorimetric method for determination of pyridine nucleotides in plant material and its use in following changes in the pyridine nucleotides during the respiration climacteric in apples. Phytochem. 7: 337-353.

54. Richmond, A. and J. B. Biale. 1966. Protein synthesis in avocado fruit tissue. Arch. Biochem. Biophys. 115: 211-214.

55. Sacher, J. A. 1966. Permeability characteristics and amino acid incorporation during senescence (ripening) of banana tissue. Plant Physiol. 41: 701-708.

56. Shipway, M. R. 1971. Effect of supraoptimal carbon dioxide concentrations on mitochondrial activity of 'Richared Delicious' apples. Ph. D. thesis 1971. University of Massachusetts, Amherst.

57. Spencer, M. 1965. Fruit ripening. In: Plant Biochemistry, ed. J. Bonner and J. E. Varner. Academic Press. New York. pp. 793-822.

58. Stoner, C. D. and J. B. Hanson. 1966. Swelling and contraction of corn mitochondria. Plant Physiol. 41: 255-266.

59. Wardlaw, C. W. and E. R. Leonard. 1935. Res. St. Imp. Coll. Trop. Agri. Mem. No. 1.
(Original not seen, cited by Hulme et al. 1971)

60. Wiskich, J. T., R. E. Young and J. B. Biale. 1964. Metabolic processes in cytoplasmic particles of the avocado fruit. VI. Controlled oxidations and coupled phosphorylation. Plant Physiol. 39: 312-322.

61. Young, R. E. and J. B. Biale. 1967. Phosphorylation in avocado fruit slices - reduction to the respiratory climacteric. Plant Physiol. 42: 1357-1362.

A P P E N D I X

Appendix Talbe 1. Comparison of Whole Fruit Respiration and Oxidations of Different Substrates by Mitochondria from 'Lodi' Apples.

Experimental conditions as in Table 2 except that the "base" rates of oxidation are reported instead of the State IV from the second addition of ADP, and that no catalytic amount of malate was added to pyruvate.

Time at 21°C	Whole Fruit Respiration	Substrate		
		Succinate	Malate	Pyruvate
days	CO_2 mg/kg-hr	nmole O_2/min-mg protein		
0	---	68.53	7.92	7.93
1	50.80	---	---	---
2	60.68	77.93	56.96	11.08
4	70.50	61.57	48.43	4.08
5	66.35	---	---	---
6	65.81	60.52	46.07	4.70
7	56.34	---	---	---
8	51.93	47.22	51.52	7.89

Appendix Table 2. Effect of TPP on oxidations of Succinate, Malate, and Pyruvate by Mitochondria from 'Lodi' Apples.

Experimental conditions as in Appendix Table 1 except that 0.085 umole of TPP was added after the "base" rate of oxidation.

Time at 21°C	Malate		Pyruvate		Succinate	
	-TPP	+TPP	-TPP	+TPP	-TPP	+TPP
days	nmole O_2/min-mg protein					
0	7.92	11.06	7.93	12.78	68.53	68.53
2	56.96	56.96	11.08	12.34	77.93	67.16
4	48.43	53.82	4.08	9.12	61.57	59.28
6	46.07	44.23	4.70	7.37	60.52	53.22
8	51.52	44.97	7.89	8.75	47.22	---

Appendix Table 3. Effects of TPP, Arsenite and Glutamate on Oxidation of Pyruvate, by Mitochondria from 'Richared Delicious' Apples Held Different Intervals at 21°C.

Base reaction medium as in Table 2. Basal rates are for State IV oxidation. Additions of 0.085 umole of TPP, 5.1 umoles of AsO_2^-, and 5.1 umoles of glutamate were made at steady state following the preceding addition.

Time at 21°C	Additive			
	Basal	TPP	AsO_2^-	Glutamate
days	nmoles O_2/min-mg protein			
0	15.8	26.8	3.6	14.8
1	16.4	38.3	9.7	24.1
2	22.9	35.4	9.5	24.1
3	19.6	30.7	9.0	21.7
7	22.3	43.3	12.2	20.5
8	16.2	35.4	10.3	36.1
11	19.7	43.3	9.4	26.9
14	16.0	44.9	3.1	24.7
16	19.1	40.1	8.2	31.1
Average	18.7 ± 2.5	37.6 ± 5.9	8.3 ± 3.1	24.9 ± 6.0

Appendix Table 4. Effects of TPP, Arsenite and Glutamate on Oxidation of Malate by Mitochondria from 'Richared Delicious' Apples Held Different Intervals at 21°C.

Experimental conditions as in Appendix Table 3.

Time at 21°C	Additive			
	Basal	TPP	AsO_2^-	Glutamate
days	nmole O_2/min-mg protein			
0	29.7	29.7	16.2	18.0
1	49.5	52.0	28.3	21.6
2	41.2	43.7	30.1	30.1
3	33.1	35.1	19.6	23.8
7	34.9	33.5	32.1	19.9
8	42.3	40.7	26.3	18.7
11	43.3	44.1	25.6	24.3
14	46.6	35.1	21.7	22.9
16	54.0	54.0	32.5	29.7
Average	41.6±8.1	40.9±8.3	25.8±5.8	23.2±4.4

Appendix Table 5. Effects of TPP, Arsenite and Glutamate on Oxidation of Succinate by Mitochondria from 'Richared Delicious' Apples Held Different Intervals at 21°C.

Experimental conditions as in Appendix Table 3.

Time at 21°C	Additive			
	Basal	TPP	AsO_2^-	Glutamate
days	nmole O_2/min-mg protein			
0	127.2	127.2	99.5	103.0
1	110.3	99.2	92.3	76.6
2	117.4	121.5	98.5	95.1
3	109.5	105.7	85.7	71.5
7	102.0	102.0	69.8	64.2
8	94.6	89.9	66.4	52.6
11	96.9	96.9	67.9	56.6
14	93.2	93.2	60.8	60.8
16	104.1	96.9	56.6	53.9
Average	106.1±11.6	103.6±12.8	77.5±16.6	70.5±18.0

Appendix Table 6. Effects of TPP, Arsenite and Glutamate on Oxidation of NADH by Mitochondria from 'Richared Delicious' Apples Held Different Intervals at 21°C.

Experimental conditions as in Appendix Table 3.

Time at 21°C	Additive			
	Basal	TPP	AsO_2^-	Glutamate
days	nmole O_2/min-mg protein			
0	167.3	167.3	38.5	66.6
1	191.4	142.8	65.1	65.1
2	205.8	157.1	65.4	81.2
3	172.2	124.5	58.9	63.6
7	133.4	108.4	49.9	33.9
8	114.0	90.3	40.9	51.3
11	141.5	108.3	64.8	64.8
14	112.4	82.7	42.8	42.7
16	113.9	84.9	47.8	47.8
Average	150.2±35.3	118.5±31.3	52.7±10.9	57.5±14.4

A C K N O W L E D G M E N T

I would like to express my appreciation for all the people in the Department of Plant and Soil Sciences who have extended help and assistance during this study. Special thanks should go to Dr. W. J. Bramlage, my advisor, Dr. H. O. Hultin and Dr. H. V. Marsh, my thesis committee, for their helpful suggestions throughout this work and during the preparation of the manuscript. I am further indebted to Dr. Hultin for the use of some of his facilities and equipment. Sincere gratitude is due to Dr. Bramlage for his advice, guidance and encouragement; and to my parents for their love, understanding and faith in me.

MITOCHONDRIAL BEHAVIOR DURING THE RESPIRATORY CLIMACTERIC AND RIPENING OF DETACHED APPLES

A Master Thesis

By

Wing-Yee Chan

Approved as to style and content by:

W. J. Bramlage

Dr. W. J. Bramlage
Chairman of Committee

Donald N. Maynard

Dr. D. N. Maynard
Head of Department (Acting)

H. O. Hultin

Dr. H. O. Hultin
Member

Herbert V. March, Jr.

Dr. H. V. Marsh
Member

July 1973